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Cell-to-Cell Contact as an Efficient Mode of Epstein-Barr Virus Infection of Diverse Human Epithelial Cells

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We show clear evidence for direct infection of various human epithelial cells by Epstein-Barr virus (EBV) in vitro. The successful infection was achieved by using recombinant EBV (Akata strain) carrying a selective marker gene but without any other artificial operations, such as introduction of the known EBV receptor (CD21) gene or addition of polymeric immunoglobulin A against viral gp350 in culture. Of 21 human epithelial cell lines examined, 18 became infected by EBV, as ascertained by the detection of EBV-determined nuclear antigen (EBNA) 1 expression in the early period after virus exposure, and the following selection culture easily yielded a number of EBV-infected clones from 15 cell lines. None of the human fibroblasts and five nonhuman-derived cell lines examined was susceptible to the infection. By comparison, cocultivation with virus producers showed ≈800-fold-higher efficiency of infection than cell-free infection did, suggesting the significance of direct cell-to-cell contact as a mode of virus spread in vivo. Most of the epithelial cell lines infectable with EBV were negative for CD21 expression at the protein and mRNA levels. The majority of EBV-infected clones established from each cell line invariably expressed EBNA1, EBV-encoded small RNAs, rightward transcripts from the *Bam*HI-A region of the virus genome, and latent membrane protein (LMP) 2A, but not the other EBNAs or LMP1. This restricted form of latent viral gene expression, which is a central issue for understanding epithelial oncogenesis by EBV, resembled that seen in EBV-associated gastric carcinoma and LMP1-negative nasopharyngeal carcinoma. The results indicate that direct infection of epithelial cells by EBV may occur naturally in vivo, and this could be mediated by an unidentified, epithelium-specific binding receptor for EBV. The EBV convertants are viewed, at least in terms of viral gene expression, as in vitro analogs of EBV-associated epithelial tumor cells, thus facilitating analysis of an oncogenic role(s) for EBV in epithelial cells.

Epstein-Barr virus (EBV) is a human herpesvirus that exhibits strong infection tropism for B lymphocytes and immortalizes them efficiently in vitro. Upon primary infection, EBV occasionally causes infectious mononucleosis, which is characterized by T lymphocytosis reactive to proliferating B lymphocytes infected with EBV. After primary infection, irrespective of whether it is clinically overt or silent, EBV establishes the lifelong virus carrier state. In this state, EBV can be detected in two different tissues, B lymphocytes and epithelial cells, and is potentially oncogenic for both cell types, as represented by endemic Burkitt's lymphoma (BL) and undifferentiated nasopharyngeal carcinoma (NPC), respectively (reviewed in reference 46).

The interaction(s) between EBV and epithelial cells has long been of special interest based on its close association with NPC. In addition, an increasing number of studies have suggested a causal relationship between EBV and primary gastric carcinoma cases, in which all tumor cells harbor the clonal EBV genome and express several latent viral genes (11, 21, 50, 55), as do NPC cells (45, 66). The expression and/or detection of clonal EBV in nasopharyngeal dysplasia (44) and in normal or metaplastic gastric epithelium (17, 61) also implies its involvement in an initiation, or earlier, phase of epithelial tumor development. In contrast to B cells, however, in epithelial cells, neither the mechanism of EBV infection nor that of EBV-induced pathologic events is well understood, since the available in vitro infection model has been quite limited (35, 53). Such situations prompted us to exploit an efficient in vitro infection system for investigating EBV activity in epithelial cells. In our previous report, three CD21-negative gastric carcinoma cell lines were still infectable with EBV, implying CD21-independent entry of the virus into the gastric epithelium (63). The present study demonstrates that such an observation can be extended in principle to various epithelial cells of different tissue origin, which were efficiently infected by cell-to-cell contact. Furthermore, we scrutinized the expression of EBV genes in the virus-infected epithelial cells, showing that they displayed a restricted pattern of viral gene expression similar to that in gastric carcinoma cells (21, 55). Our study relied heavily on a unique system for producing a clonal EBV recombinant that carries a selective marker gene (51), but it required no other assistance for infection. Our findings may explain the spread of EBV to epithelial cells at different anatomical sites in vivo (7, 8, 11, 16, 17, 19–21, 29, 32, 36, 44, 60, 61), and the technique for generating EBV-converted epithelial cells will also be applicable to experimental infection of normal epithelium with the virus.

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MATERIALS AND METHODS

Cells. A total of 27 cell lines were used in this study (Table 1). They included 21 human respiratory, gastrointestinal, hepatobiliary, and urogenital epithelial cell lines of different tissue origin, normal human fibroblasts, and five nonhuman epithelial and fibroblast cell lines. They were grown in RPMI 1640 medium (GIBCO BRL, Rockville, Md.), Dulbecco's modified Eagle's medium (GIBCO BRL), or Ham's F-12 medium (GIBCO BRL), all of which were supplemented with 10% fetal calf serum (FCS) and antibiotics. Cultures were passaged by treating the cells with 0.1% trypsin–1 mM EDTA–phosphate-buffered saline (PBS; pH 7.2) solution and diluting them 1:10 twice a week. When necessary, cells were dislodged by treatment with 2 mM EDTA-PBS. An Akata cell clone infected with recombinant EBV (see below) was maintained in RPMI 1640 containing 10% FCS and G418 (700 μg/ml; GIBCO BRL).

TABLE 1. Cells used in this study

Cell line	Origin	Source[a]	Reference
MKN1	Human gastric adenocarcinoma	JCRB	41
NU-GC-3	Human gastric adenocarcinoma	JCRB	2
MKN74	Human gastric adenocarcinoma	JCRB	41
HuCC-T1	Human biliary carcinoma	JCRB	38
HEp-2	Human laryngeal carcinoma	S. Chiba	40
HepG2	Human hepatocellular carcinoma	M. Sakai	1
EBC-1	Human lung squamous cell carcinoma	JCRB	23
LK-2	Human lung squamous cell carcinoma	JCRB	62
PC10	Human lung squamous cell carcinoma	H. Akita	
LC-1 sq	Human lung squamous cell carcinoma	JCRB	24
RERF-LC-MS	Human lung adenocarcinoma	HSRRB	31
A549	Human lung adenocarcinoma	H. Akita	13
DLD-1	Human colon adenocarcinoma	JCRB	9
LoVo	Human colon adenocarcinoma	JCRB	10
WiDr	Human colon adenocarcinoma	JCRB	42
CaR1	Human colon adenocarcinoma	JCRB	27
NT-2	Human renal cell carcinoma	N. Shinohara	52
SMKT-R-3	Human renal cell carcinoma	N. Shinohara	39
T24	Human urinary bladder carcinoma	JCRB	4
HeLa	Human uterine cervical carcinoma	JCRB	12
293	Adenovirus E1-transformed human embryonic kidney cells	ATCC	15
MRC-5	Human embryonic lung fibroblast	ATCC	25
COS-7	Simian virus 40-transformed African green monkey kidney cells	JCRB	14
BHK	Baby hamster kidney cells	JCRB	37
Rat1	Rat fibroblast	E. Kieff	
CHO-K1	Chinese hamster ovary cells	JCRB	28
NIH 3T3	Mouse embryonic fibroblast	JCRB	26

[a] JCRB, Japanese Cancer Research Resources Bank; HSRRB, Health Science Research Resources Bank; ATCC, American Type Culture Collection.

Virus. We used recombinant Akata EBV carrying the neomycin resistance (Neo^r) gene inserted into BXLF1 by homologous recombination as described previously (52). In this paper, the recombinant Akata EBV is referred to as rEBV. rEBV-infected Akata cells, isolated by reinfection of EBV-negative Akata ($Akata^-$) cells with rEBV, had been transfected in advance with a plasmid carrying the herpes simplex virus type 1 (HSV-1) thymidine kinase (*tk*) and *gpt* genes. Cells integrated with the HSV-1 *tk* gene were subsequently selected in hypoxanthine-aminopterin-thymidine medium containing mycophenolic acid (1 μg/ml). These modified Akata cells (rEBV-infected $Akata^-$ [tk^+] cells) were convenient for cocultivation experiments (see below), because they could be completely eliminated from cultures by the addition of ganciclovir (1 μM) to $G418^+$ selection medium, resulting in the survival of only G418-resistant virus recipients (i.e., rEBV-infected epithelial cells). rEBV-infected $Akata^-$ (tk^+) cells were induced for production of infectious rEBV by surface immunoglobulin G (sIgG) cross-linking (56). Briefly, dialyzed anti-human IgG goat serum (Dako, Glostrup, Denmark) was added to cell suspension (2×10^6/ml) to give a final concentration of 0.5% (vol/vol). The cells were then incubated at 37°C for 2 h with intermittent shaking, washed twice, and resuspended (10^6/ml) in RPMI 1640 medium containing 10% FCS. After a 3-day incubation, the culture was clarified by centrifugation ($1,200 \times g$) at 4°C for 15 min. The supernatant was filtered through a 0.45-μm-pore-size membrane, divided into aliquots, and stored at −80°C until used. The sIgG cross-linking consistently induced virus replication in over 60% of rEBV-infected $Akata^-$ (tk^+) cells, as evaluated by indirect immunofluorescence for synthesis of the viral structural antigen gp350 at 24 h after cross-linking.

Infection procedures. One or 2 days before infection, epithelial cells to be used as virus recipients (Table 1) were detached by treating them with 2 mM EDTA-PBS and were seeded into a 12-well culture plate at 5×10^4 cells in 2 ml of the appropriate medium per well. On the day of infection, all culture medium was replaced with the same volume of fresh medium. We employed two different infection procedures: inoculation with cell-free rEBV and cocultivation with rEBV-infected $Akata^-$ (tk^+) cells as the virus donors. For cell-free infection, 1 ml of virus supernatant prepared as described above was added directly to the cultures. For cocultivation, after sIgG cross-linking, 1 ml of rEBV-infected $Akata^-$ (tk^+) cell suspension (5×10^5/ml) was added to the cultures. Both cultures were then incubated for 3 days at 37°C in 5% CO_2, with replacement of half of the medium with fresh medium on day 2. The FCS concentration of the culture medium was reduced to 5% during the infection period to prevent cell overgrowth. After completion of the infection step (day 3), the cocultivation cultures were gently but thoroughly washed four times with PBS to remove residual viable virus donor cells, and 2 ml of fresh medium containing 10% FCS was added again. On day 4 or 5, the cells were reseeded into 96- or 24-well plates at 10^2 to 10^4/ml per well in culture medium containing an appropriate concentration of G418 for selection (200 to 700 μg/ml).

The initial infection efficiency was assessed by EBV-determined nuclear antigen (EBNA) 1 expression. In cocultivation experiments, to discriminate EBNA1-positive recipient cells from occasional contaminated virus donor cells, we routinely performed dual immunofluorescent staining of EBNA1 and cytokeratins. Ganciclovir (1 μM) was added to the cloning cultures for the cocultivation method for the first week only.

Immunofluorescence. EBNA1 was detected by anticomplement immunofluorescence with human immune serum (titer, 1,280×) on acetone-methanol-fixed cells. EBNA2 and latent membrane protein (LMP) 1 were stained by streptavidin-biotin immunofluorescence with mouse monoclonal antibodies (MAbs) PE2 (64) and CS1-4 (47), respectively. EBV lytic infection was detected on acetone-methanol- or acetone-fixed cells by indirect immunofluorescence with the BZ1 (65) and C1 (59) MAbs, specific for the immediate-early BZLF1 protein and the viral envelope antigen, gp350, respectively. The cells were then incubated with a fluorescein isothiocyanate-labeled $F(ab')_2$ fragment of rabbit antibody to mouse IgG (Dako). Staining of cytokeratins was also done in parallel with EBNA1 staining, with a mixture of the AE1 and AE3 MAbs (Dako), followed by incubation with a rhodamine-labeled rabbit antibody to mouse Ig (Dako).

To examine the expression of CD21, cells were prepared by treatment with 2 mM EDTA-PBS at 37°C for 10 to 15 min, washed with cooled medium, and reacted with MAbs OKB7 (Ortho Diagnostics, Raritan, N.J.) and HB-5a (Becton Dickinson, Mountain View, Calif.). The second reaction was done with a fluorescein isothiocyanate-labeled $F(ab')_2$ fragment of rabbit antibody to mouse IgG, followed by flow cytometric analysis.

Southern blotting. DNA was extracted by the standard proteinase K-sodium dodecyl sulfate (SDS) method, followed by phenol-chloroform purification. The DNA samples were digested with an appropriate restriction enzyme, electrophoresed in 0.7% agarose (Takara, Otsu, Japan), and blotted onto nylon membranes (Amersham International plc, Buckinghamshire, United Kingdom) in 1 N NaOH solution by vacuum transfer. The membranes were then washed briefly with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and subjected to prehybridization for 2 h at 42°C. Hybridization was performed overnight in 50% formamide–5× SSC–5× Denhardt's solution–0.5% SDS. As probes, we used a *Bam*HI-K fragment of EBV DNA for detection of EBV, and a 1.9-kb *Xho*Ia subfragment of EBV *Eco*RI-Dhet and an *Eco*RI-I fragment for clonal analysis (45). A 1.7-kb *Ase*I fragment of the pcDNA3 vector (Invitrogen, San Diego, Calif.) was used to detect the Neo^r gene. The probes were labeled with [α-^{32}P]dCTP by random priming and purified by gel filtration. After hybridization, the membranes were washed twice in 1× SSC–0.1% SDS for 15 min at

room temperature and then in 0.1× SSC–0.1% SDS for 10 min at 65°C. Autoradiography was done overnight at −80°C.

Immunoblotting. Cell lysates were prepared as previously described (21), run on an SDS–7.5 or 10% polyacrylamide gel, and blotted onto nitrocellulose membranes (Schleicher and Schuells, Dassel, Germany), followed by overnight blocking with Tris-buffered saline containing 5% nonfat dry milk (TBS-M; pH 7.6) at 4°C. To detect EBNAs, the membranes were incubated for 2 h at room temperature with pooled human sera containing antibodies to EBNA1, -2, -3A, -3B, and -3C, which were optimally diluted (1:50 to 1:200) in TBS-M. Then the membranes were washed three times with TBS-M–0.1% Tween 20 (TBS-TM) and reacted for 30 min at room temperature with horseradish peroxidase-conjugated sheep antibody to human IgG (Amersham) (diluted 1:2,000 in TBS-M). Expression of EBNA2 and LMP1 was examined by using PE2 (diluted 1:50 in TBS) and CS1-4 (diluted 1:100 in TBS) MAbs, washing with TBS-T, and incubating with horseradish peroxidase-conjugated sheep antibody to mouse IgG (Amersham) (diluted 1:1,500 in TBS) under the same conditions as above. After the second antibody reaction, the membranes were washed five times with TBS-T, immersed in enhanced chemiluminescence solution (Amersham), and subjected to the detection step according to the manufacturer's protocol.

ISH. In situ hybridization (ISH) was performed to investigate EBV-encoded small RNA (EBER) 1 expression. First, 10^4 cells detached by trypsin treatment were dispensed into wells of an eight-chamber slide glass (Nunc-InterMed, Tokyo, Japan) and incubated until the cultures reached 60 to 80% confluency. The slides were then air dried and fixed with freshly prepared 4% paraformaldehyde–0.1 M phosphate buffer (pH 7.4) overnight at 4°C. After a brief washing with 0.1 M phosphate buffer, they were treated with proteinase K (10 to 30 μg/ml) for 20 to 30 min at 37°C. The optimal conditions for proteinase K treatment had been determined for each cell line. Details of the following procedures and probe sequence have been described previously (22).

RT-PCR. Total RNA was extracted from 5×10^5 cells by using Trizol reagent (GIBCO BRL) according to the manufacturer's protocol. For cDNA synthesis, 100 pmol of a random primer (GIBCO BRL) was added to the RNA sample, followed by heating at 94°C for 5 min and rapid chilling on ice. Reagents were added to the RNA-primer mixture to give a final concentration of 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol, 0.5 mM (each) deoxynucleoside triphosphate, 10 U of RNasin (Promega, Madison, Wis.), and 200 U of Moloney murine leukemia virus reverse transcriptase (GIBCO BRL). The reverse transcription (RT) reaction was carried out at 37°C for 60 min in a total volume of 20 μl, which was then heated at 94°C for 3 min to stop the reaction. cDNA synthesized from 250 ng of total RNA was used for each PCR. Full details of all primers and probes used to detect EBV- or CD21-specific transcripts are given elsewhere (55, 63), except for the reverse primer (5′-TTC GGTCTCCCCTAGGCCCTG-3′) used to amplify the *Bam*HI-W and -C promoter (Wp and Cp, respectively)-initiated EBNA mRNA. When this renewed primer is used, the predicted sizes of PCR products of Wp- and Cp-initiated EBNA mRNA are 240 and 302 bp, respectively. The PCR mixture consisted of 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 200 μM (each) deoxynucleoside triphosphate, 20 pmol of each primer, 2.5 U of KlenTaq DNA polymerase (Clonetech, Palo Alto, Calif.), and cDNA in a total volume of 50 μl. The mixture was subjected to 30 cycles of amplification with a model 2400 thermal cycler (Perkin-Elmer, Foster City, Calif.); each cycle consisted of 94°C for 30 s, 45 to 55°C (variable for optimal detection of each transcript) for 30 s, and 72°C for 1 min. The extension time was prolonged to 5 min in the last cycle. The integrity of the RNA was checked by the parallel amplification of β-actin mRNA. The PCR products were electrophoresed in 2.5% agarose gels and blotted onto nylon membranes. Southern hybridization was done with [γ-^{32}P]ATP end-labeled internal oligonucleotide probes.

Chromosome analysis. Cells growing in the logarithmic phase were incubated in culture medium containing demecolcine (Colcemid; 2 mg/ml) for 30 min at 37°C, treated with 75 mM KCl solution, and fixed with methanol-acetic acid. Chromosomes were banded with Giemsa stain (G banding) by the standard procedure. Karyotype analysis was performed on 20 metaphases of each cell.

RESULTS

EBV infection of various epithelial cell lines. After a 3-day exposure to EBV, adherent cells were collected and the initial efficiency of infection was examined by simultaneous immunofluorescent staining for EBNA1 and cytokeratins. In experiments using the virus supernatant, cells dually positive for EBNA1 and cytokeratins were observed in only 4 of a total of 21 human epithelial cell lines, and the proportions of double-positive cells were 0.1 to 2.1% (Table 2). On the other hand, in infection by cocultivation, double-positive cells were detectable in 18 cell lines and their percentages were consistently higher than that in cell-free infection, varying from 0.1 to 19.4% among the cell lines (Table 2).

Subsequent limiting dilution of the virus-exposed cells in selection medium produced G418-resistant clones from 15 cell lines by cocultivation and from only 3 cell lines by cell-free infection. In all the drug-resistant cell lines, EBV carriage was confirmed by EBNA1 expression (Fig. 1) and Southern blot hybridization (Fig. 2). The frequency of isolation in each cell line is shown in Table 2. Consistent with the results of EBNA1 staining, infection by the cocultivation method always gave rise to much more resistant clones than cell-free infection, e.g., an approximately 800-fold difference in NU-GC-3 cells. Furthermore, increasing the number of freeze-thaw cycles to three during preparation of cell-free virus did not enhance the infection efficiency for epithelial cells. Despite repeated trials, EBV-converted cells were not obtained from three human carcinoma lines, LK-2, LC-1 sq, and WiDr, in which the double-positive cells had been recognized several days after virus exposure (Table 2). The other three human carcinoma lines, normal human fibroblasts (MRC-5), and five nonhuman cell lines were reproducibly insusceptible to EBV infection. The number of EBV genomes carried in each EBV-converted clone was estimated to be from 3 to more than 20 copies per cell, as assessed by Southern hybridization with the EBV *Bam*HI-K (Fig. 2) or Neo^r (data not shown) probe. Hybridization with the *Xho*Ia subfragment of *Eco*RI-Dhet EBV probe yielded a band identical to that detected by the *Eco*RI-I probe in each convertant, indicating that EBV DNA was maintained as an episomal form, not integrated into the cellular DNA (data not shown).

Since an earlier study suggested that EBV has fusogenic

TABLE 2. Efficiency of EBV infection in epithelial cell lines

Cell line	Efficiency of infection by:			
	Coculture[a]		Virus supernatant	
	EBNA1$^+$ cells at days 3 to 5 (%)[b]	No. of EBV convertants per 4×10^5 initial cells	EBNA1$^+$ cells at days 3 to 5 (%)[b]	No. of EBV convertants per 4×10^5 initial cells
MKN1	2.1	50	0	0
NU-GC-3	15.0	5,320	0	6
MKN74	3.3	444	0	4
HuCC-T1	0.5	28	0	0
HepG2	17.8	255	1.5	0
HEp-2	0.6	214	0	0
EBC-1	2.6	63	0	0
LK-2	1.4	0	0	0
PC10	1.8	4	0	0
RERF-LC-MS	0.8	122	0	0
LC-1 sq	0.1	0	0	0
DLD-1	4.8	2,765	0.2	32
LoVo	19.4	468	2.1	0
WiDr	0.1	0	0	0
CaR1	0.1	4	0	0
NT-2	2.9	208	0.1	0
T24	0.7	122	0	0
293	3.3	1,360	0	0
HeLa	0	0	0	0
A549	0	0	0	0
SMKT-R-3	0	0	0	0
MRC-5	0	0	0	0
COS-7	0	0	0	0
BHK	0	0	0	0
Rat1	0	0	0	0
NIH 3T3	0	0	0	0
CHO-K	0	0	0	0

[a] The ratio of virus donors to recipients was 10:1.
[b] EBNA1-cytokeratin double-positive cells.

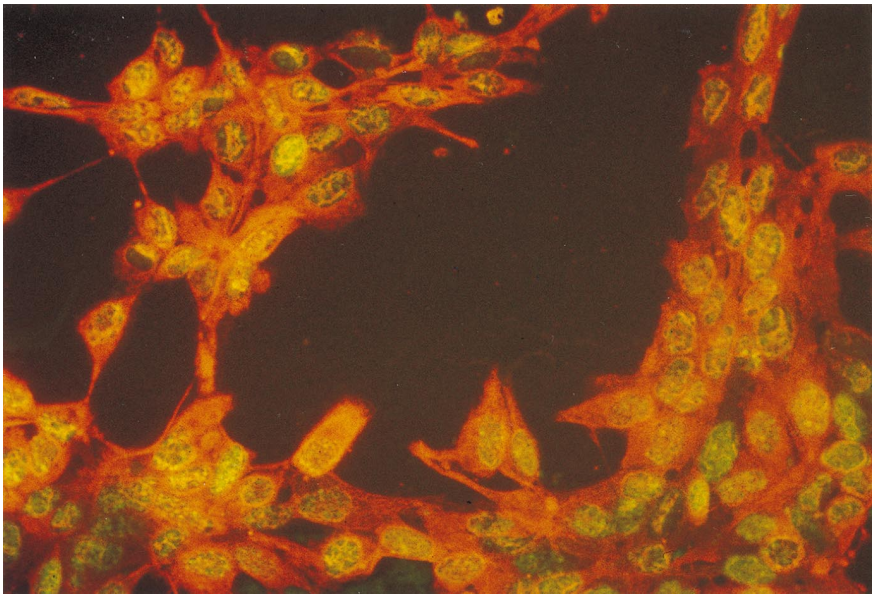

FIG. 1. Dual immunofluorescent staining of EBNA1 (green fluorescence) and cytokeratins (red fluorescence). Staining of a representative G418-resistant clone which appeared in a NU-GC-3 culture is shown. Magnification, ×400.

potential between virus-producing cells and uninfected cells, we performed chromosome analysis of the EBV convertants established through cocultivation. All the convertants had the same karyotype as their EBV-negative parent cells, indicating that EBV entry into epithelial cells did not occur via cell fusion.

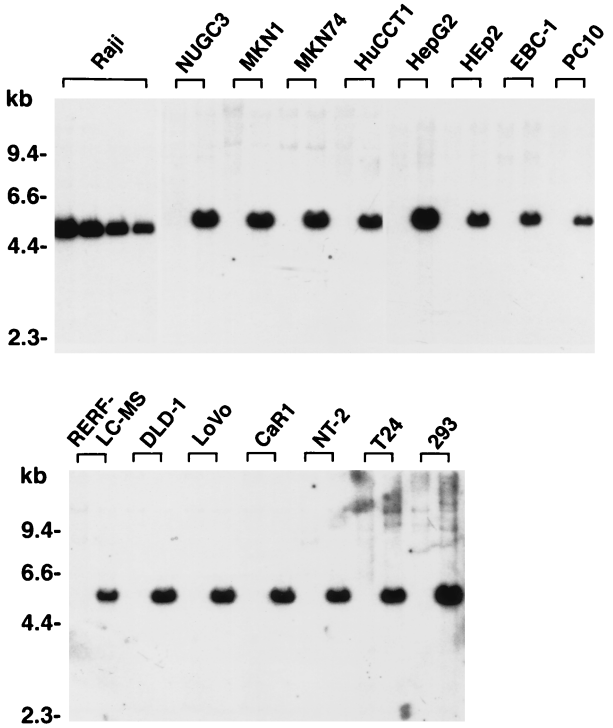

FIG. 2. Southern blot analysis of G418-resistant epithelial cells. The blots were probed with a *Bam*HI-K fragment of EBV DNA. Serially diluted samples of Raji cell DNA (2.5, 1.25, 0.63, and 0.31 μg) served as positive controls. Each of the other pairs of lanes contained 5 μg of DNA extracted from the indicated EBV-negative parent epithelial cell lines and from their G418-resistant clones (left and right lanes, respectively, of each pair). All DNA samples were digested with *Bam*HI.

EBV gene expression in epithelial cells. The ISH assay demonstrated that EBER1 was abundantly transcribed in all EBV-infected epithelial cells (Fig. 3). Immunofluorescence and immunoblot analyses revealed that most EBV-infected epithelial cells expressed EBNA1, but not EBNA2, -3A, -3B, -3C, or LMP1 protein (Fig. 4A). When the same analysis was performed on several EBV-infected clones isolated from each cell line, only a few of the clones were positive for LMP1 (Fig. 4B). Such a clonal difference was not observed for the EBNA expression status: only EBNA1 was expressed in all converted clones (data not shown). We further examined the expression of LMP2A, LMP2B, transcripts from the *Bam*HI-A region of the virus genome (BARF0), and EBNA promoter utilization in these converted cells by RT-PCR (Fig. 5). LMP2A and BARF0 mRNAs were consistently detected in all EBV-converted clones isolated from 15 cell lines. LMP2B mRNA was less frequently detectable in seven of these lines, and the level of its expression was generally lower than that of LMP2A. RT-PCR analysis also indicated that EBV-converted clones derived from eight epithelial cell lines were negative for LMP1 mRNA (data not shown). A low level of LMP1 transcripts was detected in the remaining seven epithelial cell lines, which also expressed low levels of LMP2B, consistent with the coupled transcription status of both LMPs previously reported in NPC (6). In EBV-converted clones from these seven epithelial cell lines, LMP1 protein was detected in less than 1% of the cells by immunofluorescence assay (data not shown). Thus, the absence of LMP1 expression is most likely due to blockage at the level of transcription.

Among the three known transcriptional promoters for EBNA genes, Qp was active in all EBV convertants. Transcripts initiated from Cp and/or Wp were faintly detected in several cell lines, at much lower levels than those from Qp (Fig. 5). Spontaneous activation of the lytic cycle-specific BZLF1 and gp350 genes was detected by immunofluorescence staining in <0.5 and $<0.2\%$, respectively, of acutely infected cells (4 to 14 days postinfection) and stably infected clones. However,

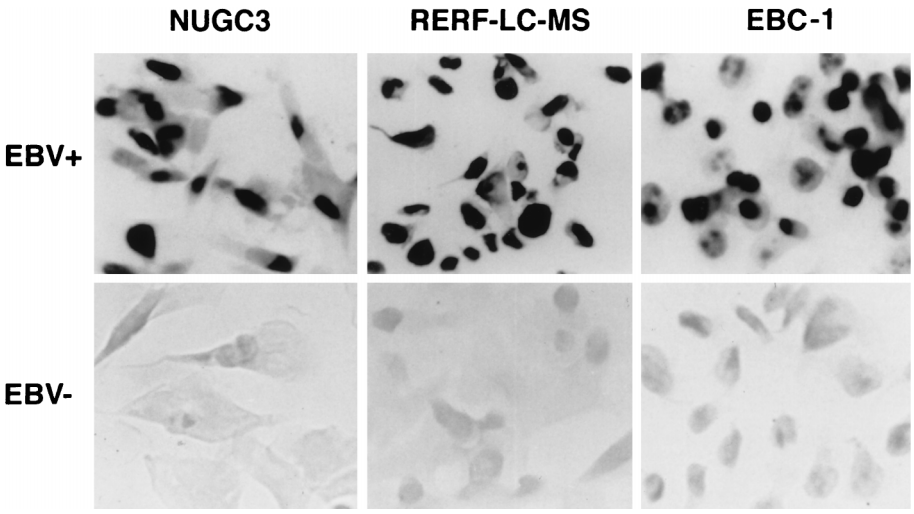

FIG. 3. In situ detection of EBER1 expression in rEBV-infected epithelial cell lines. rEBV-converted NU-GC-3, RERF-LC-MS, and EBC-1 clones are shown in the top row, and their parental cells are shown in the bottom row. Strong nuclear signals can be seen in the EBV convertants but not in their parental cells. Magnification, ×600.

there were some exceptions in which about 5 and 0.8 to 2% of EBV-infected cell clones derived from MKN74, HEp-2, and DLD-1 cells were positive for BZLF1 and gp350, respectively.

CD21 expression in epithelial cells. To investigate whether the successful infection of epithelial cells by EBV was attributable to CD21, the parental cells of EBV convertants were examined for expression of CD21 at the transcriptional and protein levels. Flow cytometric analysis showed that these cell lines were clearly negative for CD21, although DLD-1 showed weakly positive staining (Fig. 6A). Mostly compatible with the flow cytometric results, the RT-PCR assay revealed CD21-specific transcripts in 4 of the 18 cell lines that were found to

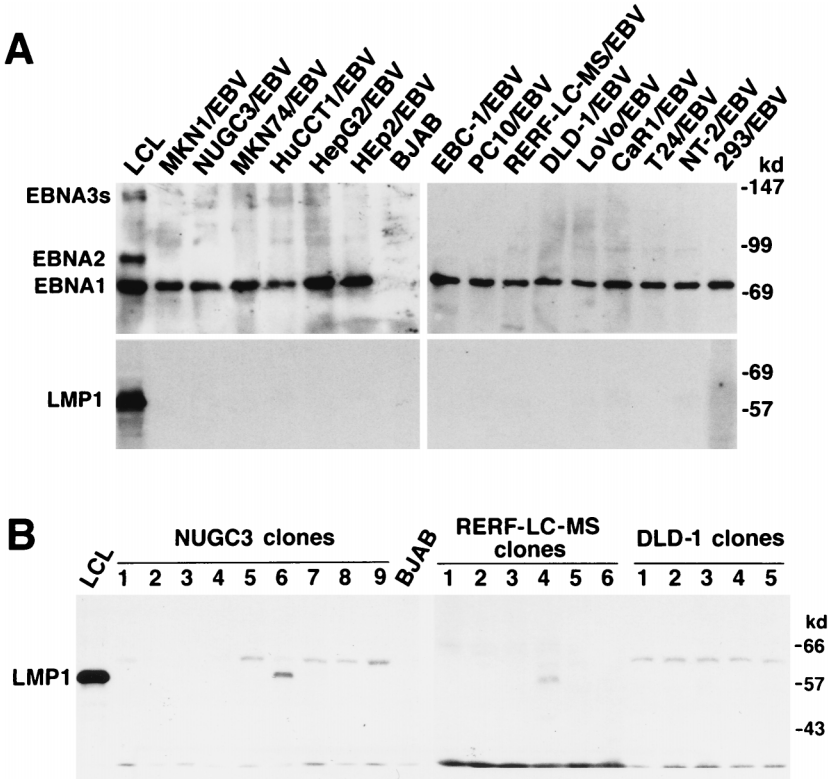

FIG. 4. Immunoblot analysis of EBV latent gene expression in virus-infected epithelial cells. (A) Expression of EBNAs and LMP1. Protein blots were probed with a pool of EBV-seropositive human sera for EBNAs and with CS1-4 MAbs for LMP1. Lysates extracted from 10^5 cells of each rEBV-infected clone were used per lane. Lane labels indicate infected clones: MKN1/EBV, for example, indicates an rEBV-infected MKN1 clone. (B) Analysis of the clonal difference in LMP1 expression. Representative results for rEBV-infected clones from NU-GC-3, RERF-LC-MS, and DLD-1 cells are shown. Only two clones (NU-GC-3 clone 6 and RERF-LC-MS clone 4) were positive for LMP1. LCL, B-lymphoblastoid cell lines immortalized with rEBV as a positive control; BJAB, EBV-negative B-cell control.

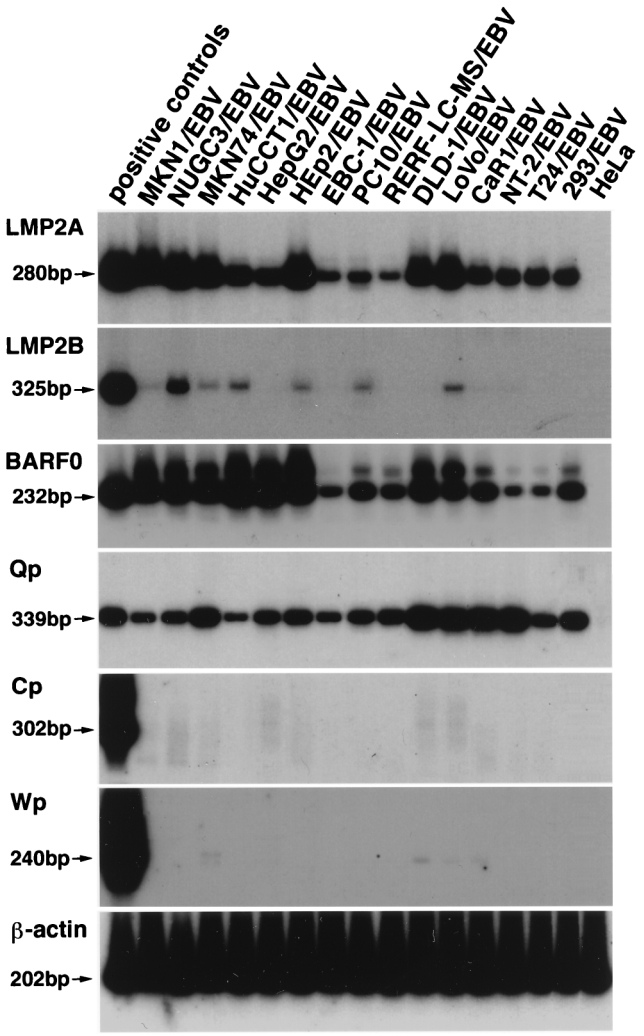

FIG. 5. RT-PCR analysis of EBV latent gene expression in virus-infected epithelial cells. LCL, B-lymphoblastoid cell lines immortalized with rEBV, was used as a positive control for detection of LMP2A, LMP2B, BARF0, and Cp- or Wp-initiated EBNA mRNAs. rEBV-infected Akata^{-} (tk^{+}) cells were used as a positive control for detection of Qp-initiated EBNA mRNA. HeLa cells served as a negative control. Labels on the lanes are explained in the legend to Fig. 4.

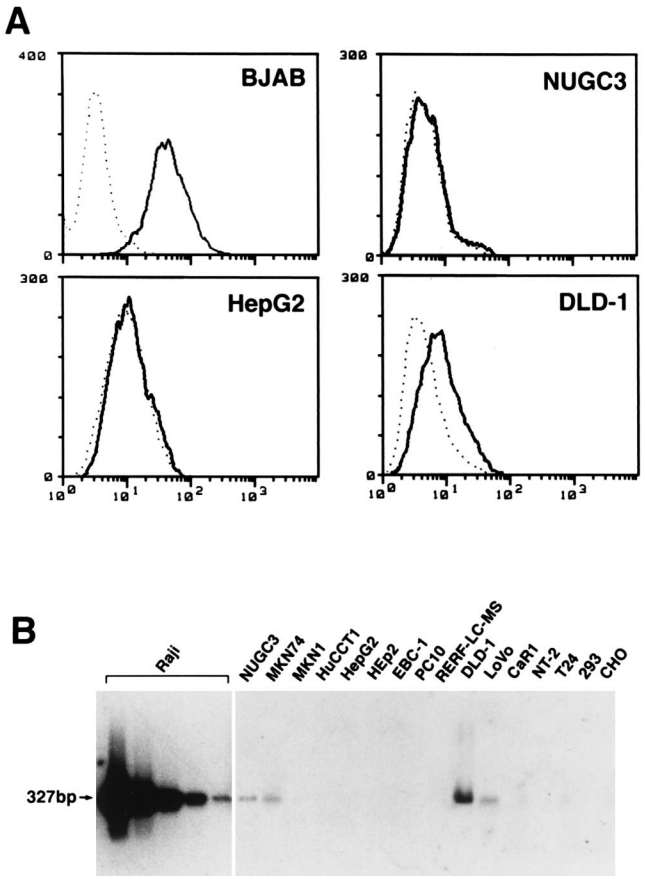

FIG. 6. CD21 expression in epithelial cells. (A) Flow cytometric analysis. Results for three representative epithelial cell lines highly susceptible to EBV infection are shown. A CD21-positive clone of BJAB (an EBV-negative B-cell line) was used as a positive control. The solid and dotted lines indicate staining with a mixture of anti-CD21 MAbs (HB5a and OKB7) and isotype controls, respectively. The vertical axis denotes the number of cells counted, and the horizontal axis denotes fluorescence intensity (log scale). (B) RT-PCR analysis. Transcription of CD21-specific mRNA was examined in all epithelial cell lines from which EBV convertants were isolated. Each lane of Raji represents amplification of cDNAs generated from serially fourfold-diluted total RNA (250, 62.5, 15.6, 4.0, and 1.0 ng of total RNA from left to right) from Raji cells as positive controls. The other lanes contained amplified products of 250 ng of total RNA from each epithelial cell line used in the study.

be susceptible to EBV infection, but not in the others (Fig. 6B). Among the four cell lines positive for CD21 transcription, DLD-1 cells expressed a relatively large amount of the mRNA compared with NU-GC-3, MKN74, and LoVo cells. However, even CD21-specific mRNA expression in DLD-1 was much lower than that of control Raji cells (Fig. 6B).

DISCUSSION

The acknowledged difficulty in investigating the oncogenic potential of EBV in epithelial cells is the lack of an efficient infection system, which is ascribed mainly to the lack of CD21 expression. Epithelial cells, including normal ones, however, inherently permit EBV infection, provided that the barrier is overcome by CD21 expression via gene transfer (35) or membrane implantation (49). We previously succeeded, without any such artificial manipulations, in infecting gastric carcinoma cell lines with EBV (63), research motivated by accumulated clinical evidence for the association of EBV with gastric carcinoma (11, 21, 50, 55). The present study further extended those results to a variety of epithelial cells derived from different anatomical sites. The successful EBV infection of a wider range of epithelial cells than used to be thought possible has considerable relevance to the in vivo detection of the virus in epithelia elsewhere than in the nasopharynx and stomach, such as the respiratory tract (19, 29, 36) or the hepatobiliary system (20).

Most of the epithelial cell lines infected by EBV in the present study had negative or extremely low CD21 expression, suggesting that, consistent with our previous results (63), an unidentified epithelium-specific binding receptor(s) distinct from CD21 mediates the infection. In addition, our data imply that the novel receptor(s) may commonly exist in human, but probably not in other mammalian, epithelium. Human leukocyte antigen class II (HLA-DR) has recently been identified as a cofactor for EBV infection of B cells (34). However, HLA-DR-negative epithelial cell lines were still infectable in our study (e.g., cell line NU-GC-3), indicating that the molecule is dispensable for the infection of epithelial cells. Although the exact reason why cocultivation showed much higher infection

efficiency than cell-free infection is unknown, this is also the case with other viruses, such as human T-cell leukemia virus type I (5). Assuming that the novel receptor has lower affinity for EBV than does CD21, close cell-to-cell contact could augment the accessibility of virions to cells, thus increasing the chance of binding to the cell surface, followed by viropexis.

Sixbey and Yao pioneered research into CD21-independent EBV entry into epithelial cells in vitro—infection mediated by polymeric IgA (pIgA) against viral gp350 (53). This explicit phenomenon can easily explain the involvement of EBV infection in the development of NPC and possibly of gastric carcinoma, which are typically preceded and accompanied by the appearance of virus-specific IgA in serum (11, 18, 21, 33). Our data, however, also represent a conceivable in vivo situation in which EBV infection of epithelial cells can occur naturally without the mediation of gp350-specific pIgA. In this context, cell-to-cell contact with virus producers is presumably another efficient mode of EBV infection in vivo, which may be supported by the fact that EBV-infected but nondiseased epithelial regions are detected in healthy virus carriers who have no serum IgA against viral antigens (8, 36, 60). In such a situation, virus donors are most likely EBV-infected B cells migrating into the epithelial stroma or intraepithelial space (57, 58). Since a population of EBV-infected epithelial cells spontaneously enters into the lytic cycle in vitro (references 30, 35, 54, and 63 and the present study) as well as in vivo (7, 16, 32, 36), the epithelial cells themselves are considered to be an occasional source for the intercellular spread of the virus. Although the mechanism of infection by cell fusion between EBV-infected lymphocytes and EBV receptor (CD21)-negative cells is also reported to be implicated (3, 8), the possibility is negated based on our own observations that (i) several cell lines were infectable with virus supernatant, (ii) EBV-infected epithelial cells were able to grow in the presence of ganciclovir (the HSV-1 *tk* gene exists only in virus donor cells), (iii) polykaryocytes indicative of cell fusion (3) were not obvious during the culture, and (iv) the converted cells had karyotypes identical to those of their parent cells. With regard to the virus strain-dependent difference in infection efficiency previously suggested (30, 35), our preliminary results indicate that the B95-8 strain of EBV is also infectious to epithelial cells. However, we have not determined the relative infection efficiencies of Akata and B95-8 viruses. In accord with our series of results, an attempt to infect primary epithelia by rEBV is one of our current projects.

All EBV-infected cells presented in this report uniformly displayed a restricted pattern of latent viral gene expression. They expressed EBNA1, EBERs, LMP2A, and BARF0 exclusively, while the other latent genes were largely negative, though a clear clonal difference was observed in LMP1 expression by immunoblotting. These results are compatible with promoter utilization for EBNA transcription: transcripts from Qp were constitutively detected, whereas Cp and Wp were inactive, with the exception of very weak Cp- or Wp-specific signals in several cell lines. This format of latent viral gene expression, which differs from the conventional EBV latency of types I and II seen in BL and most NPC cases (46), respectively, makes our epithelial convertants analogous to EBV-positive gastric carcinoma cells (21, 55) or a subgroup (LMP1-negative) of NPC cells (66). Therefore, the convertants can serve as useful in vitro models for studying the oncogenic potential of EBV in an epithelial background.

The regulation of latent infection gene expression, especially of the EBNA genes, is a key aspect in the development of EBV-associated malignancies, because EBV-specific cytotoxic T lymphocytes are known to mainly recognize all EBNA proteins other than EBNA1, resulting in the complete elimination of virus-infected cells (46). Recent investigations indicate that some of the interferon regulatory factors (IRFs) bind to a regulatory *cis* element of Qp (QRE-2) and activate (IRF-1 and -2) or repress (IRF-7) Qp in BL cells that show type I latency (43, 48). It is thus necessary to examine whether the IRF-dependent regulation of Qp activity is also present in epithelial cells. The panel of epithelial cells used in our research will provide suitable materials for this objective and also for studies on other unknown interactions between EBV and epithelial cells.

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